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Biophysical targeting of adenovirus vectors for gene therapy

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Advances in understanding the interaction of animal viruses with their cognate receptors has led to improvements in the development of cell-specific, targeted viral vectors. Research strategies to generate safe, non-inflammatory viral vectors that are capable of delivering a therapeutic gene to a specific population of cells are currently underway in many laboratories. One approach in the utilization of this cell targeting activity is to ablate the natural interaction of the virus with its native receptor, although this is not an absolute requirement. The initial development of 'viral targeting strategies' was based on the view that by modifying the viral protein/receptor interaction, it would be possible to redirect virus vectors to new host cells. As the understanding of virus/cell interactions increased it was observed, however, that many viruses can use different entry mechanisms for cell attachment and penetration.

Adenovirus vectors have been used extensively for the delivery of genes to cells. The entry mechanism for adenoviruses into cells has recently been studied and is relatively well understood, however, there are many aspects of cell receptor/virus interactions, which have still to be elucidated. The single high-affinity receptor on mammalian cells for adenovirus type 5 is recognized as the coxsackie and adenovirus receptor. However, in the absence of coxsackie and adenovirus receptor other receptors are used. A thorough understanding of the biology of adenoviruses is essential in the further development of their use as vectors for cell targeting. One strategy is to modify the viral capsid, either through coating the surface using bispecific antibodies, or by chemically crosslinking the targeting ligand onto the virion surface. Another approach is to genetically modify the virus by incorporating the targeting ligand into the viral 'spike' (fiber) protein. This involves manipulating the adenovirus genome and generating a new targeting ligand on the surface of the fiber protein using recombinant DNA technology. The penton base protein has also received attention as a means of directing adenoviruses via insertion of novel targeting ligands.

Keywords Adenovirus, fiber protein, gene delivery, targeting, viral vector

Introduction

First generation adenovirus (Ad) vectors can be constructed so that they have severely attenuated replication ability by removing the *E1* gene function. This prevents the replication of the recombinant Ad, except in certain mammalian helper cell lines, where *E1* is provided in *trans* [1]. Ad vectors offer many advantages as vectors for clinical applications in gene therapy. These include: (i) their natural ability to infect a wide range of cell types and achieve gene expression irrespective of cellular replication; (ii) their ability to multiply infect a single cell; (iii) the ability to generate high titer virus stocks; (iv) high vector stability; (v) their ability to deliver a foreign gene into the nucleus of a cell; and (vi) a suitably sized cloning capacity for the introduction of foreign genes. The fact that many cell types are capable of infection by Ad vectors is also a major drawback in their use as gene therapy vectors. Successful targeting of Ad vectors will potentially have a number of advantages. Firstly, cell type-specific targeting of the viral vector will result in transgene expression only in the cell type of choice, thus permitting systemic administration whereby the virus would be localized to specific cell types, tissues or organs [2]. Secondly, the inflammatory and immune responses against the vector may be reduced, as these are thought to derive from uptake into antigen-presenting cells and from virus binding to native receptors, promoting the concomitant stimulation of cytokines [3]. Inflammation due to activation of the transcription factor NFκB by the virion has been observed following infection using a vector with no transgene (but only at high multiplicity of infection (MOI) > 1000 pfu per cell). This inflammatory response was also observed after the virus had been inactivated using UV irradiation [4]. Such inflammatory responses result in rapid clearance of the Ad vector and thereby reduce its therapeutic potential. The need to eliminate this response has, in part, promoted the move towards vectors with more adenoviral genes removed. Second generation vectors with deletions in the *E1*, *E2B* and *E4* regions exhibit decreased immunogenicity when compared with the first generation *E1a/E1b*-deleted vectors [5]. Such deletions require the provision in *trans* of the viral genes using a helper cell line. Further deletions of viral genes have been achieved using the *Cre-Lox* system to remove approximately 25 kb of the viral genome producing 'gutless vectors' [6,7]. These vectors require the use of an *E1*-deleted helper virus to propagate virus particles in a permissive cell line, although a potential drawback is contamination with helper virus at a level of up to 1% [8]. Despite this potential limitation, gutless vectors have been constructed which encode full-length dystrophin [8] and the cystic fibrosis transmembrane conductance regulator proteins [9], which have been used *in vitro* to infect dystrophin-deficient muscle cells and primary cultures of human airway epithelial cells derived from a cystic fibrosis (CF) patient, respectively.

Adenovirus biology

Virion structure

Adenoviruses are non-enveloped regular icosahedral-shaped viruses. The diameter of the virion along the 5-fold symmetry axis is 73 nm [10,11]. The icosahedral shell (capsid) of the virion is composed of 252 subunits (capsomeres) comprising 240 hexons and 12 pentons, containing viral DNA. The internal surface of the hexon is

mainly hydrophobic and faces the DNA core [12]. Each of the 12 pentons at the vertices of the icosahedron contains a penton base, which is surrounded by five hexons. Each virion consists of 12 vertices with an antenna-like 'fiber' protruding from each vertex [13,14]. The fiber protein is a trimeric protein comprising two regions, a shaft, which is derived from the N-terminus of the protein and is variable in length between different Ad serotypes, and a terminal knob, which is derived from the C-terminus of the protein and is approximately 21 kDa in Ad type 5 (Ad5).

Receptor-mediated entry through the adenovirus fiber

The Ad replicative pathway occurs via a series of steps that ensure synchrony of infection and production of new virion particles. Virus entry is accomplished via receptor-mediated endocytosis with uncoating occurring in the acidified endosome. Previous studies on the Ad fiber knob region demonstrated that it was responsible for early attachment and receptor interaction via the coxsackie and adenovirus receptor (CAR) [15••,16••], and the initial binding of the fiber to the CAR receptor acts as an anchor at the cell surface. The second stage of viral entry involves an interaction between the RGD (Arg-Gly-Asp) sequence present within the penton base of the capsid and α_v-integrins present on the cell surface [17]. Other studies have reported that the MHC class I α2 domain on human epithelial and B-lymphoblastoid cells acts as a high affinity receptor for Ad5, with human fibronectin type III as an auxiliary receptor [18]. However, when both CAR and MHC class I α2 domain were co-expressed on the surface of hamster cells, Ad5 fiber bound only to CAR, suggesting that CAR is the single high affinity receptor for this serotype. MHC class I is expressed on most cells and it has been shown that MHC class I knockout mice are still susceptible to Ad infection, indicating the use of an alternative receptor [19]. It is also known that different Ad serotypes interact with different cell surface cofactors for attachment when there is little or no surface expression of CAR [20,21], and the α_v integrins may mediate some Ad-binding in the absence of CAR [3]. Although CAR interactions have been shown to bind most Ad serotypes, they will not bind those from serotypes 3, 7 and 35. The third stage of viral infection involves replication which occurs as a 'cascade reaction', beginning with transcription of the Ad early genes. During Ad transcription, host cell DNA and protein synthesis is inhibited, and within 36 h, synthesis of virions is complete, the infected cell has doubled its DNA and protein content, and the resulting cell lysis releases new particles, the large majority of which are non-infectious [22].

Approaches to adenovirus targeting

Early studies of viral targeting relied on the premise that a modified virus could be specifically targeted to a cell type by exploiting the understanding of interactions between Ad vectors and host cells. Although to some extent this premise is true, the entry pathway for Ad vectors is complex and auxiliary receptors and cellular cofactors may be required for attachment and internalization in the absence of the cognate viral receptor. Given that the interaction between the fiber and RGD motif within the penton base are important for virus entry, the majority of targeting strategies have centered around modification of these two proteins, particularly the fiber knob region. Other strategies have included the modification of the virus surface by physical coating, or the use of tissue-specific promoters to drive the transgene (transcriptional targeting). A successful targeting strategy must allow specific interaction only with a receptor on the desired cell type. Targeting may be achieved by an expansion of the natural tropism either by modification of the fiber, penton base or capsid.

Polymer approach

A number of different groups have reported the use of polymers to produce coated Ad particles. These complexes have been used either to target Ad, or Ad vectors have been incorporated into DNA/polymer complexes to improve cellular transfection efficiency. Researchers have described the use of Ad particles to improve the transfection of adipocytes, monocytes and hepatoma cells [32,33]. DNA/polymer complexes were produced using the polymers lipofectamine and polyethylenamine (PEI) followed by addition of Ad to the transfecting mixture. The authors report that Ad particles improve transfection some 140- to 300-fold when compared to PEI alone. They concluded that involvement of the Ad receptor and/or α_v-integrins was probably responsible for the observed improvements, by promoting endosomal release. The formation of DNA/polymer/Ad complexes using PEI has also been described by Baker *et al* [34]. Obviously, the formation of complexes between cationic polymers and Ad does not endow any specific targeting ability onto the virus. In the cases described above, any tissue-specific gene expression was achieved *in vitro* using cell type-specific promoters. The use of crosslinking complexes between Ad vectors and cationic polymers has also been used to improve transfection of airway epithelial cells for use in CF gene therapy [35]. The formation of DNA/Ad complexes were reported with a variety of different cationic polymers and lipids including poly-L-lysine, histone, DEAE-dextran, PEI, spermine, DC-Chol, dioleoyl phosphatidylethanolamine (DOPE) and dioctadecylamidoglycylspermine (DOGS). Using primary cultures of human airway epithelial cells, the complexes were shown to correct the electrophysiological abnormalities that characterize CF epithelia. Once again, no element of direct targeting is endowed upon the virus by virtue of complex formation with polymers, since targeting is achieved simply by administration into the airways.

The use of polymers to coat virus particles has two distinct advantages. Firstly, it may be used to mask capsid proteins and thereby prevent interaction with the CAR receptor and α_v-integrins and the stimulation of a humoral immune response. Secondly, coating with an activated polymer provides a mechanism by which novel targeting ligands may be attached to the virion, thus enabling its redirection. The use of activated polyethylene glycol (PEG) (tresylmonomethoxypolyethylene glycol, TMPEG), which covalently attached to the surface of the Ad via reaction with surface-exposed lysine residues, was described [36]. Results indicate that PEGylation is sufficient to protect Ad particles from the effects of neutralizing antibodies in the lungs of mice, with high antibody titers to Ad [36].

Coupling of surface PEGylated Ad particles to targeting peptides derived from a phage display library is a novel method of redirecting Ad particles. The use of phage display

to select peptides with binding activity for ciliated airway epithelial cells, which lack sufficient receptors for effective *in vivo* transduction by Ad has been reported [37]. The selected peptide with the most effective binding was coupled to the surface of the Ad particle via the bifunctional PEG molecule. The authors report that entry into airway epithelia is increased above that of a PEGylated virus without the attached targeting peptide, and that entry is independent of CAR-binding, since excess fiber protein had minimal effect on transduction. Transduction of HeLa cells, however, was competed by excess fiber, indicating that the fiber proteins are not masked by the concentration of activated PEG used in these experiments. A similar approach to expand the tropism of Ad vectors for hematopoietic stem cells has recently been described [38]. Photoactivatable biotin was employed to attach the targeting ligand stem cell factor (SCF) via an avidin bridge to the capsid of the Ad. Transduction assays indicated that an increase in expression of the luciferase transgene of between 440- and 2350-fold was observed and that expression could be competed with an excess of the receptor, c-Kit. Cells that were non-permissive for the novel targeting ligand showed no increase when compared with cells transduced with unmodified vector. The authors have also applied the technology more generically by coupling antireceptor antibodies to the biotinylated Ad particles and studying their ability to direct gene transfer. More modest levels of gene expression were observed when compared with the SCF targeted virus but it does, however, indicate that this system is applicable to several different directional gene transfer routes. Direct biotinylation of Ad particles does not prevent the fiber or penton base interacting with CAR receptors or α_v-integrins, respectively.

Modifications to the capsid

Many cell types can be transduced using Ad vectors; cells that cannot be readily transduced exhibit either low numbers of or a total absence of CAR receptors. The inability of Ad vectors to infect other cells may be due to non-specific reasons, ie, the presence of mucous or the inaccessibility of CAR (it is not on the apical surface of the cell). Although the fiber protein has been demonstrated to provide the primary cellular interaction, fiberless mutants of Ad have been constructed and enter cells via the interaction between the RGD motif in the penton base and the cell surface α_v-integrins [23,24]. As expected, the infectivity of these fiberless particles was severely reduced, however, binding to the cellular integrins was normal. Moreover, modified fibers could also be incorporated by the use of packaging cell lines providing the fiber gene in *trans*. Since fiberless particles can be propagated and retain infectivity, modifications to the capsid provide a potential method of targeting to specific or novel cell types. Different approaches have been identified utilizing antibodies, direct crosslinking to capsid proteins and coating with polymers.

Ad can be targeted to endothelial and smooth muscle cells (SMCs), which have low or undetectable levels of CAR receptors, using bispecific antibodies comprising a monoclonal antibody to α_v-integrins chemically linked to a monoclonal antibody to FLAG peptide epitope (DYKDDDDK) [25]. The FLAG epitope was incorporated into the Ad penton base protein. Complexing the new AdFLAG vector with the bispecific antibody increased transduction of human endothelial cells by between 7- and 9-fold when compared with uncomplexed AdFLAG. Interestingly, transduction of 293 cells was unaffected in the presence of the bispecific antibody, indicating that transduction efficiency is not necessarily reduced when fiber-mediated cell binding is circumvented.

Several groups have manipulated the penton base protein with replacement of the RGD motif [26]. A contrasting approach has been the construction of a series of nine 'isogenic' viruses in which the hypervariable region 5 of the hexon base was replaced with a polio virus epitope (DNPASTTNKDK) flanked by differing peptide spacers [27]. Although none of these modifications affected the replicative capacity of the Ad, there was a marked effect on epitope recognition by a cognate monoclonal antibody. Vigne and colleagues constructed a virus in which the RGD motif was introduced into the hexon base protein, aiming to determine whether transduction of cell types with low density or absence of CAR receptors could be obtained. Although the hexon base had not previously been implicated in virus entry, the modified virus exhibited significantly increased transduction of human SMCs *in vitro*. Competition experiments, in which an excess of fiber knob protein was incorporated in cellular infection assays, indicated that the presence of the RGD motif within the hexon base endowed a viral route of infection in addition to the normal attachment pathway.

Modification of the capsid proteins also provides a means of propagating viruses with modified fiber proteins that do not interact with the CAR receptor [28-30]. Inactivation of the CAR receptor-binding ability is beneficial for the construction of Ad vectors with systemic utility, however, vectors with CAR receptor-binding ablated fiber proteins require the construction of novel helper cell lines for the propagation of virus particles. Modifications to the penton or hexon base proteins clearly provide a means to propagate virus particles using the 293 or PerC6 cell lines [31]. Einfield and colleagues described the construction of two viruses in which hemagglutinin (HA) was incorporated into the penton base or the HI loop of the fiber protein [30]. Both virus constructs could be propagated by transduction of 293 cells that had been transfected with an expression construct encoding an anti-HA single chain antibody (scFv fragment), 293-HA. Insertion of HA into the penton base mediated transduction of Chinese hamster ovary (CHO) cells. In these cells (CHO-HA), transduction was inhibited only by an excess of HA and not fiber protein, indicating that transduction was dependent only on the interaction between pseudoreceptor and the HA within the penton base, as CHO cells do not possess CAR receptors.

Modifications to the fiber

Antibody-based approaches

Modification of the fiber protein has probably received the most attention when targeting strategies have been devised, since the fiber protein is involved in the primary cell-binding interaction with the CAR receptor. Immunological modifications to the fiber have been based on the premise that the use of a neutralizing antiknob antibody will ablate CAR binding and serve as a means of attachment for a novel cell-binding ligand. There have been numerous reports

describing variations upon this theme. Researchers have described the use of a neutralizing Fab fragment chemically crosslinked with the basic fibroblast growth factor (FGF2) for targeting to a number of tumor cell lines expressing fibroblast growth factor receptors, including Kaposi's sarcoma cells [39-41]. Other groups used a bispecific antibody approach that linked a monoclonal antiknob Fab fragment to a monoclonal anti-epidermal growth factor receptor (EGFR) using a bifunctional crosslinking agent [42]. The rationale behind this approach was that glioma cells probably exhibit heterogeneous expression of CAR receptors and α_v-integrins, but not EGFR. Targeting by EFGR was predicted to both improve specificity and increase transduction of cells expressing low levels of CAR receptors. Specific fiber-independent transduction of both primary glioma cells and cell lines was demonstrated with EGFR targeting. Similar approaches using bispecific antibodies have been used to conjugate a monoclonal anti-epithelial cell adhesion molecule (EpCAM) onto a neutralizing antifiber knob antibody for increased transduction of cancer cells [43]. A second example was demonstrated by conjugating a monoclonal anti-CD40 antibody onto a neutralizing antifiber knob antibody for improved transduction of dendritic cells [44]. Watkins *et al* developed the immunological approach further with the production of a phage library displaying single chain antibodies derived from the spleen of a mouse immunized using Ad knob protein [45]. A neutralizing scFvs was isolated which was genetically fused to epidermal growth factor (EGF), producing an 'adenobody'. This was then used to target Ad with enhanced transduction of cells expressing EGFR.

Bispecific antibodies have also been constructed against the AdFLAG vector in which the FLAG epitope has been inserted into the hypervariable region of the penton base coat protein [25]. These bispecific antibodies have been chemically linked to anti-CD3 and anti-E-selectin [46,47] for targeting to T-cells and vascular endothelial cells, respectively. Whilst these are not modifications of the fiber, they have been included as they are immunologically-mediated targeting methods. Transduction of T-cells was increased 500-fold over the control vector by using the anti-CD3/anti-FLAG conjugate, and transduction of cultured endothelial cells was increased by 20-fold.

The immunological targeting approaches described above have all been used to improve the ability of Ad vectors to transduce cell types that possess few or no CAR receptors and/or α_v-integrins, and they have been evaluated using either established cell lines or primary cell cultures. Preliminary studies in our laboratory have generated encouraging results using a cell targeting strategy with modifications to the fiber protein. The objective was to generate targeting ligands for the delivery of Ad vectors encoding therapeutic genes directly to terminally differentiated neurons. First generation replication-deficient Ad vectors expressing the reporter gene *β-galactosidase* (RAd35) were developed [48], and cell targeting was determined using a predicted targeting ligand derived from tetanus toxin, which was chemically conjugated directly onto a monospecific polyclonal antifiber antibody; the presence of reporter gene expression was then identified in N18 and Vero cells. Specific receptors for tetanus toxin were present on N18 cells but not on Vero cells [49], and as predicted there was no protein expression in Vero cells. A dose-dependent increase in reporter gene expression was detected in N18 cells which correlated with increasing concentration of the targeting ligand conjugate [Charlton S, unpublished data]. It is thought that this fiber modification strategy has successfully identified a specific motif with cell-specific targeting capabilities.

Genetic approaches

Modification of the fiber gene using recombinant technology is likely to be the most successful long-term strategy for retargeting Ad, as stable modified particles may be reliably produced. Genetic modifications to the fiber fall into two groups; those that attach a novel ligand onto the C-terminus of the fiber protein and those that incorporate a ligand coding sequence into the fiber gene, most commonly within the HI loop (for a description of the fiber structure see [50,51]). To date, none of the insertions within the fiber gene have ablated CAR-binding, as the residues involved in CAR receptor interaction had not been identified at the time these constructs were made. Recently, researchers have described a number of site-directed fiber mutations that abolish CAR-binding [52•,53•,54••].

Possibly the simplest way in which the Ad tropism has been modified is by production of fiber/capsid chimeras, utilizing the natural spectrum of tropism elicited by the various serotypes of human and animal adenoviruses. Gall and colleagues described a chimeric virus where a fiber gene from Ad7 was used to replace the fiber from Ad5 [55]. Although this exchange of fiber genes did not dramatically alter the tropism, the intention was to demonstrate the feasibility of such an approach. Interestingly, *in vivo* use of the chimera vector combining the Ad7 fiber/Ad5 capsid indicated that antifiber antibodies did not significantly contribute to the overall neutralizing immune response to the Ad. Other researchers have also described an Ad3/Ad5 fiber chimera (the knob region of which was derived from Ad3 and the shaft from Ad5) and noted that the receptor recognition profile was altered when compared with an Ad5 vector [56]. The development of a novel two-plasmid system for propagation of such chimeric vectors has also been reported. Researchers described an Ad5 capsid with a chimeric Ad3 knob/Ad5 shaft fiber [57]. Transduction results obtained with the vector possessing a fiber chimera indicated that some cell types (THP-1, MRC-5 and FaDu) were more readily transduced, whilst others (human coronary artery endothelial cells) were more efficiently transduced by the Ad5 fiber vector. Other workers reported the construction of a chimeric virus with improved transduction efficiency for hematopoietic stem cells [58]. Different Ad serotypes were screened for interaction with non-cycling human CD34+ cells and K562 cells, with particular attention to the ability of the viruses to attach, internalize and replicate. Ad35 B was found to possess the highest tropism for CD34+ cells, and a chimeric vector based on the capsid of Ad5 and the short shafted fiber from Ad35 was constructed. *In vitro* infection studies indicated that the chimeric virus exhibited a 2- to 4-fold increase in transduction of CD34+ cells when compared with an Ad5 vector.

Another fiber modification approach has been the inclusion (at the C-terminus of the fiber protein) of additional targeting ligands. Researchers constructed an Ad vector containing a string of seven lysines at the C-terminus [59]. The virus, denoted AdZ.F (pK7), was targeted to heparan sulfate-containing receptors that are broadly expressed on many cell types. Gene transfer to alveolar epithelial, endothelial, peripheral blood macrophages and SMCs *in vitro* using this vector was found to increase between 5- and 500-fold when compared with control Ad5 vector. *In vivo*, SMCs (porcine iliac arteries) exhibited increased transduction compared to that obtained with the control vector. The same vector was used to determine the transduction efficiency in skeletal muscle cells, primarily to assess its suitability for the delivery of the human dystrophin gene [60]. *In vivo*, mature skeletal muscle cells were transduced with 4-fold higher efficiency than the control unmodified Ad5 vector. The efficiency of transduction of cells in both normal and inflamed colon by the AdZ.F (pK7)-modified virus was compared with an unmodified Ad5 *in vivo* [61]. Again, increased levels of reporter gene expression between 10- and 40-fold were observed in spleen T-cells and murine lamina propria mononuclear cells. Gonzalez and colleagues utilized the AdZ.F (pK7) vector to overcome the low efficacy of infection for fresh myeloma cells exhibited by unmodified Ad vectors [62,63]. At an MOI of 12, only myeloma cells were significantly transduced and gene transfer was inhibited by excess heparin, confirming that heparan sulfate-containing receptors were mediating the gene transfer events.

The construction of an Ad5 with a stretch of 20 lysine residues at the C-terminus of the fiber protein has also been described [64]. This mutation greatly enhanced transduction efficiency in four human glioma cell lines examined, however, no significant improvement to the transduction efficiency was observed with human fibroblasts or various tumor cell lines, including melanoma, prostate, esophageal or pancreatic. It was also demonstrated that 10- and 100-fold increased transduction of malignant glioma cell lines was obtained using the AdZ.F (pK7) vector [65].

The HI loop of the fiber protein has recently been utilized for the insertion of relatively large peptides (up to approximately 80 amino acids), whilst retaining the ability of the fiber to trimerize. The number of different epitopes introduced thus far into the HI loop are relatively few, but include the FLAG peptide [66•] and RGD motif [67]. Introduction of the FLAG peptide into the HI loop indicated that firstly, the fiber structure was not adversely affected, and secondly that the epitope was accessible, such that targeting ligands introduced at this site would be available for potential interaction with a cell receptor. These findings were elegantly exploited by introducing an RGD motif into the HI loop of Ad5 [68••]. This vector has potential utility for targeting cells that do not possess CAR receptors but do have α_v-integrins. The Ad5RGD vector was inoculated via the lateral tail vein in mice and tissue distribution of the *luciferase* reporter gene studied. Expression of luciferase was higher with the AdRGD vector than an unmodified Ad5 control vector (in the spleen, kidney and liver), whereas in the heart, expression was reduced c mpared with the control vector. The AdRGD has been examined for utility as a gene transfer vector for cancer cells [69]. AdRGD transduced cell lines derived from squamous cell carcinoma of the head and neck with a 4-fold higher efficiency than the unmodified control vector. Interestingly, gene expression could not be competed with excess fiber knob protein indicating that binding to the cell surface was mediated by the cell surface α_v-integrins, rather than CAR (to which the unmodified vector control was bound).

Conclusions

Currently, the aim of researchers is to design Ad vectors that target specific cells which do not induce an inflammatory response against the vector. The approaches to genetically-modify fiber proteins have all introduced targeting ligands that permit viral transduction of cells that lack sufficient CAR receptors for significant levels of gene expression to be obtained. Vectors modified by inclusion of such targeting ligands into the HI loop or at the C-terminus of the fiber will presumably retain their native tropism if delivered systemically. The recent elucidation of the residues within the fiber which interact with CAR now opens up the potential to both ablate CAR-binding and introduce novel tissue-specific targeting ligands, although with the caveat that new helper cell lines will be required to propagate such virus particles.

It will also be important to specifically address safety issues before these approaches are used successfully in the clinic; these include the prevention of pro-inflammatory responses to the vector and the assessment of optimal routes of virus inoculation. Although the route of gene delivery via the intrahepatic artery using high-titered Ad vectors can cause liver toxicity, it is unclear whether such severe accumulation of virus in the liver would occur via a different route of delivery in humans. Further research is still required to reveal optimal strategies for repeated targeted gene delivery and long-term gene expression in the presence of immunological memory responses to either the vector or to the transgene. Refinements of the vector by removing more of the viral genome ('gutless vectors') to eliminate immune responses to the vector backbone may be critical in overcoming the safety and toxicity issues for clinical use. A second requirement will be to overcome the promiscuous nature of Ad vectors to ensure that the targeting moiety is cell-specific. Further improvements in vector design will make it possible to administer the vector by systemic injection, with the targeting moiety endowing upon the Ad the ability to localize to specific cells or organs within the body. This review has highlighted some of the current modalities in the development of Ad vectors for targeted gene delivery. The tragic circumstances surrounding the death in 1999 of Jesse Gelsinger in the US following a gene therapy trial using Ad vectors as a delivery vehicle for therapeutic genes have led to an independent investigation into the cause of his death [70]. Although human gene therapy trials are strictly regulated, any adverse events during a trial still underscore the need for continued fundamental research into virus interactions with the human immune system and the role of virus-mediated cytokines.

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